Development and Validation of Stability Indicating RP-HPLC Method for the Estimation of Methotrexate and Folic Acid in Bulk and Tablet Dosage Form

Kusuma Jogi1, Mandava Basaveswara Rao2 and Rundraraju Ramesh Raju*
1. Dept., of Chemistry, Acharya Nagarjuna University, Nagarjuna Nagar, Guntur(Dist), Andhra Pradesh, India.
2. Department of Chemistry, Krishna University, Machilipatnam, Krishna(Dist), Andhra Pradesh, India.

Abstract
An isocratic, reverse phase liquid chromatographic method was developed for the validation of stability of Methotrexate and Folic Acid in bulk and tablet dosage form. Methotrexate is used to treat cancer, autoimmune diseases, ectopic pregnancy, and for medical abortion. Folic acid is used as a supplement during pregnancy to prevent neural tube defects. In this paper, a simple and reliable HPLC method was developed and validated for the evaluation of Methotrexate and folic acid. A Phenomenx C-18 analytical column, 4.6 mm × 250 mm packed with 5 µm particle size containing mixture of Acetonitrile : Orthophosphoric acid (0.1%) (45:55, v/v) was used. The flow rate was 1.0 mL min⁻¹ and the UV detection was performed at 215 nm. The retention times of Methotrexate and Folic Acid were 6.4 min and 2.8 min respectively. Linearity range of Methotrexate and Folic Acid were in the range of 37.5 - 112.5 mg mL⁻¹ and 1 - 12.5 mg mL⁻¹ respectively. The proposed method was validated for linearity, accuracy, precision, robustness of Methotrexate and folic acid drug. The RSD values for all parameters were found to be less than 2, which indicates the validity of method and results obtained by this method are in fair agreement. Finally this method can be used for better analysis and pharmaceutical formulations Methotrexate and folic acid drug.

Key words: Methotrexate and Folic acid, Validation, Evaluation and formulation.

1. Introduction
Methotrexate is one of the most effective and widely used medications for treating rheumatoid arthritis (RA) and other inflammatory types of arthritis. It’s also one of the safest arthritis drugs, insist rheumatologists, despite a common misconception among many patients and even some primary care physicians that methotrexate is highly toxic.

Confusion about this important medication’s safety profile seems to exist because it is also used – in much higher doses – for treating some forms of cancer. Most patients who use methotrexate to treat their inflammatory arthritis take between 10 and 25 milligrams (mg) per week. By contrast, the doses used to treat leukemia and certain other types of cancer may be hundreds of times larger.

That’s not to suggest that taking methotrexate is risk-free. A 2009 review of 21 studies found that 73 percent of RA patients who used the medication experienced at least one side effect. Yet the study indicates that most of these problems were relatively minor. What’s more, doctors who prescribe methotrexate for arthritis say that following a few simple steps can make this drug even safer to use.

Understanding how methotrexate works helps explain why it can cause unwanted effects. Researchers originally developed methotrexate in the 1940s as a cancer drug. It stops malignant (or cancerous) cells from rapidly multiplying and spreading by blocking their access to folate, a form of vitamin B, which these cells need to survive.

Unfortunately, depleting the body of folate can affect healthy cells, too, especially those in the gastrointestinal (GI) tract, mouth, hair follicles and liver, says Prabha Ranganathan, MD, an associate professor of medicine in the division of rheumatology at Washington University School of Medicine.

GI problems such as nausea and vomiting are the most common side effects associated with
methotrexate, affecting between 20 and 65 percent of RA patients who take the drug. While hair loss is a relatively uncommon side effect in patients who take methotrexate at such doses, up to one third develop mouth ulcers, or sores. Many also complain of headaches, fatigue and an overall “blah” feeling – sometimes called “methotrexate fog” – that can occur a day after receiving a dose of methotrexate (which is taken in pill form or injected once a week).

The good news: These side effects can often be short-circuited by taking a folic acid supplement.

Folic acid is the synthetic form of folate. One study found that RA patients on methotrexate who took folic acid supplements lowered the risk of GI problems and mouth sores by 79 percent.

Dr. Ranganathan recommends taking 1 mg of folic acid daily, though for convenience some other physicians instruct patients to pop a single 5 mg dose once a week. (Some doctors recommend taking folic acid 24 hours after receiving a dose of methotrexate; ask physician for complete instructions on using folic acid supplements).

A few additional steps may help prevent or relieve GI and oral problems:

Split the dose. Most arthritis patients take methotrexate orally, in a dose consisting of several pills. Some find that splitting the dose eases GI side effects; take half the pills in the morning and the other half 12 hours later, preferably with food.

Ask about medication. For very severe stomach queasiness, your doctor can prescribe an anti-nausea drug such as ondansetron (Zofran), says pharmacist James Bennett of Children’s Hospital in Boston.

Swap your pills. When nothing else helps, switching from oral methotrexate to the injectable version can eliminate GI distress.

Try a rinse. To relieve painful mouth sores, a salt-water rinse or special mouthwash containing lidocaine (a pain reliever) may help, says Bennett.

Methotrexate chemically known as \( (2S)-2-[(4-\{(2,4-Diaminopterinyl) methyl \} (methyl) amino} benzoyl] amino} pentanedioic acid \) (Figure:1). Molecular formula \( C_{20}H_{22}N_{8}O_{5} \)

Folic Acid chemically known as \( (2S)-2-[(4-\{(2,4-Diaminopterinyl) methyl \} (methyl) amino} benzoyl] amino} pentanedioicacid\) (Figure:2). Molecular formula \( C_{19}H_{19}N_{7}O_{6} \)

2. Experimental
2.1 Chemicals and reagents
All the reagents used in the experimental work were of analytical grade. HPLC grade water was prepared by Milli-Q reverse osmosis (Millipore, Bedford, USA) and meets European Pharma- copoeia requirements. Acetonitrile and ortho-phosphoric acid (Sigma–Aldrich, Merk and Rankem) were used for preparing the mobile phase. Mobile Phase was used as solvent.

Working standards of Methotrexate and folic acid were provided by Glenmark Pharmaceuticals (Mahape, Navi Mumbai). Methotrexate and folic acid was checked by comparison with European Pharmacopoeia CRS standards. Formulation was obtained from.

2.2 Chromatographic conditions (instrumentation and analytical conditions)
An Alliance 2695 (Waters, USA) chromatographic system was used, equipped with a Quaternary pump, and waters 2996 photo diode array detector. Phenomenex C18 column, auto sampler thermostat and degasser. Chromatographic software Empower was used for data collection and processing Separations were performed using Phenomenex C-18 analytical column, 4.6 mm × 250 mm packed with 5 µm particle size. A 1m long steel capillary with 0.25 mm
internal diameter, was inserted between the injection system and the entrance of the column, and injection volume was 10 µL. Separations and simultaneous determination of Methotrexate and folic acid were performed using the mixture of Acetonitrile : Orthophosphoric acid (0.1%) (45:55, v/v) as a mobile phase. Mobile phase was filtered through a 0.45 µm Millipore filter. The flow rate was 1.0 mL min⁻¹ and the UV detection was performed at 215 nm.

3. Preparation of solutions
3.1 Standard solution preparation
Stock standard solution of 75 mg mL⁻¹ of Methotrexate and 10 mg mL⁻¹ was prepared by accurately weighing approximately 7.5 mg of Methotrexate and 1 mg of folic acid into a 10 mL volumetric flask and making up to volume with solvent.

3.2 Assay sample preparation
Weigh 10 tablets of Mext-7.5F were taken average weight crush to powder form taken into one tablet equivalent weight were transferred into a suitable volumetric flask. The volume of the volumetric flask was completed with the appropriated solvent and mixed. An accurately measured volume of this solution was quantitatively diluted with solvent.

4. Validation procedure
Chromatographic separation was optimized in the aim to obtain a resolution above 1.5 between all components, with the respect of stationary and mobile phase compositions, flow rate, sample volume, detection wavelength and temperature.

The method was validated for linearity, range, precision (repeatability and intermediated precision), specificity, limit of quantization, limit of detection, robustness and forced degradation.

4.1 Linearity and range
Standard calibration curves were prepared with five calibrators over a concentration range of 37.5–112.5 mg mL⁻¹ for Methotrexate and 1–12.5 mg mL⁻¹ for folic acid. The data of peak area versus drug concentration were treated by linear least square regression analysis. The standard curves were evaluated for linearity.

4.2 Precision
The precision of the assay was studied with respect to both repeatability and intermediated precision. Repeatability was calculated from six replicate injections of freshly prepared solution in the same equipment on the same day. Repetability for Methotrexate and folic acid was realized with a 70 and 10 µg mL⁻¹ solution. The experiment was repeated by assaysing freshly prepared solution at the same concentration on 2 additionally consecutive days to determine intermediate precision. Precision was expressed by the % of the relative standard deviation (R.S.D.) of the analyte peaks.

4.3 Specificity
Specificity of a method can be defined as absence of any interference at retention times of peaks of interest, and was evaluated by observing the chromatograms of blank samples and samples spiked with Methotrexate and folic acid. The variable number of excipient used in generic versions of Methotrexate and folic acid, as well as the lack of information in the composition of some generic formulations makes it difficult to assess selectivity by traditional analysis comparison with a placebo solution.

4.4 Limits of detection and quantization
Limits of detection (LOD) and limits of quantization (LOQ) were provided and calculation was made with the following equations:

\[
3.3 \sigma
\]
\[
\text{LOD} = \frac{3.3 \sigma}{S}
\]
\[
10 \sigma
\]
\[
\text{LOQ} = \frac{10 \sigma}{S}
\]

When σ was the standard deviation of the response (estimated from the standard deviation of y-intercepts or regression lines) and S was the slope of the standard curve.
4.5 Sensitivity
The sensitivity (6σ) of an analytical method is defined by the minimum variation that requires to be applied to the magnitude measured in order to obtain a significant variation in the signal measured.

4.6 Robustness
Robustness of method was investigated by varying the chromatographic conditions such as change of flow rate(±20%), organic content in mobile phase (± 2%), wavelength of detection (± 5%). Robustness of the developed method was indicated by the overall %RSD between the data at each variable condition.

4.7. Forced degradation
Forced degradation should be no interference between the peaks obtained for the chromatogram of forced degradation preparations. The degradation peaks should be well separated from each other and the resolution between the peaks should be at least 1.0 and the peak purity of the principal peaks shall pass.

4.8 Stability
Stability by preparing the analytical solution and injecting at periodic intervals of 24 hours to 48 hours at 3 to 4 hour intervals depending on the instrument utilization and sequence of injection.

5. Results and discussion
In this paper, we developed the reverse phased column procedure for a suitable method for the pharmaceutical analysis of Methotrexate and folic acid drug and tablets. A typical chromatogram obtained by using the mobile phase. The precision, accuracy and forced degradation of the method was determined from Methotrexate and folic acid dosage form and obtained. Inter and intra-day studies were performed in three concentrations of the drug was reported on three consecutive days.

6. METHOD VALIDATION
The method was validated for linearity, precision, accuracy, robustness, ruggedness, forced degradation and stability of the method was studied by the Methotrexate and folic acid.

Linearity was prepared in the range of 7.5-112.5 µg/ml and 1-15 µg/ml solutions are analyzed through the high pressure liquid chromatographic technique. The peak area were plotted against concentration was subjected to linear plots (Figure:4 and 5).

![Figure 4: Linearity plot for Methotrexate](image)

![Figure 5: Linearity plot for Folic Acid](image)

Precision of this method was studied in inter day and intra day variation. The precision of intraday studies of six different concentration of the drug was repeated thrice in a day and in the inter day variation studies of six different concentration of the drug was repeated on three consecutive days. The developed method was found to be precise as the percentage of RSD values for inter-day and intra-day precision studies were found to be less than 2%. Good recoveries (98 - 100%) of the drug were obtained at each added concentration, indicating that the method was accurate.
Table 1: Recovery of Methotrexate drug

<table>
<thead>
<tr>
<th>Amount of Methotrexate drug mg/ml</th>
<th>Recovery Solution (area) mAU</th>
<th>% drug recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.75</td>
<td>2170462</td>
<td>100.3</td>
</tr>
<tr>
<td>7.50</td>
<td>4610242</td>
<td>100.8</td>
</tr>
<tr>
<td>11.25</td>
<td>6607028</td>
<td>100.1</td>
</tr>
</tbody>
</table>

Table 2: Recovery of Folic Acid drug

<table>
<thead>
<tr>
<th>Amount of Folic Acid drug mg/ml</th>
<th>Recovery Solution (area) mAU</th>
<th>% drug recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.5</td>
<td>737718</td>
<td>100.5</td>
</tr>
<tr>
<td>1.0</td>
<td>1605350</td>
<td>100.2</td>
</tr>
<tr>
<td>1.5</td>
<td>2224795</td>
<td>100.7</td>
</tr>
</tbody>
</table>

LOD and LOQ minimum concentration level at which the analyte can be reliably detected (LOD) Methotrexate and folic acid, Methotrexate and folic acid drugs are 1.87 and 3.75 µg/ml, 0.25 and 0.50 µg/ml respectively. The chromatographic data as shown below fig. no’s 09 and 10.

Table 3: Results of force degradation studies

<table>
<thead>
<tr>
<th>Stress Condition/duration/solution</th>
<th>Degradation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acid degradation (0.5 N HCl, 1 hr)</td>
<td>25%</td>
</tr>
<tr>
<td>Alkaline degradation (0.5 N NaOH, 1 hr)</td>
<td>22%</td>
</tr>
<tr>
<td>Oxidative degradation (30 % H2O2,80°C for 10 min)</td>
<td>28%</td>
</tr>
<tr>
<td>Reduction Degradation (10% Sod.Bisul, 1hr)</td>
<td>24%</td>
</tr>
<tr>
<td>Thermal degradation (Solid sample, 80°C, 3 hr)</td>
<td>23%</td>
</tr>
<tr>
<td>Photolytic Degradation (sample expose sun light 6 hr)</td>
<td>27%</td>
</tr>
<tr>
<td>Hydralysis Degradation</td>
<td>22%</td>
</tr>
</tbody>
</table>

Forced degradation study was observed that upon treatment of Methotrexate and folic acid with different strengths of base (0.05 N and 0.5 N NaOH), acid (0.05 N, 0.5 N and 1 N HCl) and hydrogen peroxide and Thermal and Photolytic (20 %) the degradation was observed in (Table 3). Further it is important to note that from the chromatograms (Figure 11 to 17), it is evident that although the degraded peaks are observed. The Methotrexate and folic acid stable under the applied stress conditions like Thermal, acid and alkaline and oxidative degradation states.
Robustness of the method small changes in chromatographic conditions such as change in flow rate (± 20%), organic content in mobile phase (± 2%), pH (±0.2) and wavelength of detection (± 5%) studied to determine the robustness method for the analysis of Methotrexate and folic acid. The influence of changes in chromatographic parameters are shown in table 4. The chromatographic data figure no’s 18 to 25.

<table>
<thead>
<tr>
<th>Change in parameter</th>
<th>% RSD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Flow (0.8 ml/min)</td>
<td>0.65</td>
</tr>
<tr>
<td>Flow (1.2 ml/min)</td>
<td>0.92</td>
</tr>
<tr>
<td>Wavelength (220 nm)</td>
<td>0.74</td>
</tr>
<tr>
<td>Wavelength (210 nm)</td>
<td>0.56</td>
</tr>
<tr>
<td>Organic phase composition (+2%)</td>
<td>0.24</td>
</tr>
<tr>
<td>Organic phase composition (-)</td>
<td>0.22</td>
</tr>
<tr>
<td>pH Variation (+0.2)</td>
<td>0.36</td>
</tr>
<tr>
<td>pH Variation (-0.2)</td>
<td>0.48</td>
</tr>
</tbody>
</table>
CONCLUSIONS

The developed method is accurate, precise and reliable for the analysis of Methotrexate and folic acid in pharmaceutical formulations. This method was validated for linearity, accuracy, precision, robustness of Methotrexate and folic acid drug. The RSD values for all parameters were found to be less than 2, which indicates the validity of the method and results obtained by this method are in fair agreement. Finally this method can be used for better analysis and pharmaceutical formulations Methotrexate and folic acid drug.

References


